20 percent formamide gives the clearest pattern of the multigene bovine and porcine leukocyte interferon gene families.

C3 cont

Fig. 14 depicts a Southern hybridization of four different bovine genomic DNA phage recombinants digested with EcoRI, BamHI or HindIII and hybridized with a ^{32}P-labelled human leukocyte gene probe. Clone 83 yields two hybridizing fragments with each restriction enzyme.

Fig. 15A shows a portion of the nucleotide sequence from the plasmid subclone p83BamHI1.9kb as well as the deduced amino acid sequence for the bovine leukocyte interferon coded therein. The signal peptide is represented by amino acid residues S1 through S23.

Fig. 15B shows the nucleotide sequence and deduced amino acid sequence for a second bovine leukocyte interferon (α2) from the plasmid subclone p67EcoRI 3.2 kb.

Fig. 16 is a schematic diagram of the construction of the bovine leukocyte interferon expression plasmid pBoIFN-α1trp55. The starting materials are the trp expression vector pdeltaRIsrc and the Bam HI fragment from the plasmid subclone p83BamHI1.9kb.

Fig. 17 is a schematic of the construction of plasmid pR1.--

On page 21, in line 31, after "(1982)." please insert:

--U.S. 438,128 relates to the isolation and identification of DNA sequences encoding animal interferons and to the construction of recombinant DNA expression vehicles containing such DNA sequences operably linked to expression-effecting promoter sequences and to the expression vehicles so constructed.

C4

The following detailed description is illustrative of the invention for the preparation, via recombinant DNA technology, of the various animal interferons embraced, and sets forth generally applicable methodology for the preparation of particular, bovine leukocyte interferons. The method is described with respect to a bacterial system.

A. Isolation of Bovine DNA

For the purpose of constructing an animal gene library, high molecular weight DNA was isolated from animal tissue by a modification of the Blin and Stafford procedure (63), randomly fragmented with respect to gene locus, and size fractionated to obtain 15-20 kilobase fragments for cloning into a lambda phage vector (64).

Frozen tissue, for example bovine pancreas, was ground to a fine powder in liquid nitrogen and solubilized in 0.25 M EDTA, 1 percent Sarkosyl, 0.1 mg/ml Proteinase K (25 ml/gram tissue) at 50° C for 3 hours. The viscous solution obtained was deproteinized by three phenol and one chloroform extractions, dialysed against 50 mM Tris-HCl (pH8), 10 mM EDTA, 10 mM NaCl and digested with heat-treated pancreatic ribonuclease (0.1 mg/ml) for 2 hours at 37°C. After phenol and ether extraction, the DNA was precipitated with two volumes of ethanol, washed in 95 percent ethanol, lyophilized and redissolved in TE buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA) overnight at 4°C at a final concentration of 1-2 mg/ml. The final DNA preparation was greater than 100 kilobases in length as determined by electrophoresis on a 0.5 percent neutral agarose gel.

B. Partial Endonuclease Digestion and Size Fractionation of Bovine DNA

Aliquots (0.1 mg) of bovine DNA were digested with 1.25, 2.5, 5 and 10 units of Sau3A at 37°C for 60 minutes in a reaction (1 ml) containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM dithiothreitol. Incubations were stopped by adding EDTA to 25 mM, phenol and ether extracted, made 0.3 M in sodium acetate (pH 5.2) and precipitated with 3 volumes of ethanol. The DNA was redissolved in TE buffer at 68°C and sedimented through a 10-40 percent linear sucrose gradient (64) in a Beckman SW 27 rotor at 27,000 rpm for 22 hours at 20°C. Fractions (0.5 ml) were analyzed on a 0.5 percent gel using Eco R1-digested Charon 4A (64a) DNA as a molecular weight standard. Those fractions containing 15-20

kilobase DNA fragments were combined, precipitated with ethanol and redissolved in TE buffer.

C. Construction of the Bovine Genomic DNA Library

The 15-20 kb bovine DNA nonlimit digest was cloned into a lambda Charon 30 A vector (65) having G-A-T-C sticky ends generated by removal of the two internal Bam HI fragments of the phage. Charon 30 A was grown in E. coli strain DP 50 SupF (ATCC No. 39061, deposited March 5, 1982) in NZYDT broth, concentrated by polyethylene glycol precipitation and purified by CsCl density gradient centrifugation (66). Phage DNA was prepared by extracting the purified phage twice with phenol, once with phenol and ether, and concentrating the DNA by ethanol precipitation.

For preparation of the end fragments of Charon 30A, 50 micrograms of phage DNA was annealed for 2 hours at 42°C in 0.25 ml of 50 mM Tris-HCl (pH 8), 10 mM $MgCl_2$ and 0.15 M NaCl, digested to completion with Bam HI, phenol and ether extracted, and sedimented through a 10 to 40 percent sucrose gradient as described above. Fractions containing the 32 kb annealed arms of the phage were combined and ethanol precipitated.

The purified Charon 30 A arms (6 micrograms) were reannealed at 42°C for 2 hours, combined with 0.3 micrograms of 15-20 kb bovine DNA and 400 units of phage T4 polynucleotide ligase and incubated overnight at 12°C in a 0.075 ml reaction containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 20 mM dithiothreitol and 50 micrograms/ml bovine serum albumin. The ligated DNA mixture was then packaged into mature lambda phage particles using an in vitro lambda packaging system (67).

The components of this system-sonic extract (SE), freeze-thaw lysate (FTL), protein A, and buffers A and M1-were prepared as described (67). Three microliter aliquots of the ligated DNA mixture were incubated with 15 microliters of Buffer A, 2 microliters of Buffer M1, 10 microliters of SE and 1 microliter of protein A for 45 minutes at 27°C. The FTL was thawed on ice for 45 minutes, combined with 0.1 volumes of Buffer M1, centrifuged at

35,000 rpm at 4°C for 25 minutes, and 0.075 ml aliquots of the supernatant were added to the above reaction. After an additional 2 hours of incubation at 27°C, a small aliquot of the packaging reaction was titered on strain DP 50 SupF, supra. This procedure yielded a total of approximately 1.1x 10^6 independent bovine DNA recombinants. The remainder of the packaging mixture was amplified by a plate-lysate method (65) by plating out the recombinants on DP 50 SupF at a density of 10,000 plaque-forming units per 15 cm NZYDT agar plate.

D. Screening of the Phage Library for Bovine Interferon Genes

The strategy used to identify phage recombinants carrying bovine interferon genes consisted in detecting nucleotide homology with radioactive probes prepared from cloned human leukocyte (68,69), fibroblast (70) and immune (71) interferon genes. Hybridization conditions were established with Southern blots (72) of genomic animal DNA. Five micrograms each of high molecular weight DNA (prepared as described above) from human placenta, bovine pancreas and pig submaxillary gland were digested to completion with Eco RI, electrophoresed on a 0.5 percent agarose gel and transferred to nitrocellulose paper (72). A 32P-labelled DNA probe was prepared from a 570 base-pair Eco R1 fragment containing the protein coding region of the mature human leukocyte interferon A/D hybrid at the Bgl II restriction site (73) by standard procedures (74). Each nitrocellulose filter was prehybridized at 42°C overnight in 5XSSC (70), 50 mM sodium phosphate (pH 6.5), 0.1 mg/ml sonicated salmon sperm DNA, 5x Denhardt's solution (75), 0.1 percent sodium dodecyl sulfate, 0.1 percent sodium pyrophosphate that contained either 10 percent, 20 percent, or 30 percent formamide, and then hybridized with 100x10^6 counts per minute of the labelled probe in the same solution containing 10 percent sodium dextran sulfate (76). After an overnight incubation at 42°C, the filters were washed 4 times in 2XSSC, 0.1 percent SDS at room temperature, once in 2XSSC and then exposed to Kodak XR-5 x-ray film with Dupont Cronex intensifying

screens overnight. As seen in Figure 13, a number of hybridizing bands are most readily detected in the bovine and porcine DNA digests when 20 percent formamide is present in the hybridization. This result provides evidence for a multigene family of leukocyte interferon genes in cow and pig analogous to that previously demonstrated in humans (70,77). The same hybridization conditions were therefore employed to screen for interferon genes in the bovine DNA library.

500,000 recombinant phage were plated out on DP 50 SupF at a density of 10,000 pfu/15 cm plate, and duplicate nitrocellulose filter replicas were prepared for each plate by the method of Benton and Davis (78). The filters were hybridized with the human LeIF gene probe as described above. Ninety-six duplicate hybridizing plaques were obtained which gave strong signals upon repeated screening.

The bovine library was further screened for fibroblast and immune interferon genes. Probes were made from a 502 base-pair Xba I-Bgl III fragment containing the entire mature human fibroblast interferon gene (70), and a 318 base-pair Alu I fragment (containing amino acids 12-116) and 190 base-pair Mbo II fragment (containing amino acids 99-162) from the mature coding region of the human immune interferon gene (71). Hybridization of 1.2×10^6 recombinant phage yielded a total of 26 bovine fibroblast and 10 bovine immune interferon clones.

E. Characterization of the Recombinant Phage

Phage DNA was prepared (as described above) from 12 recombinants which hybridized with the human leukocyte interferon probe. Each DNA was digested singly and in combination with Eco R1, Bam HI and Hind III, electrophoresed on a 0.5 percent agarose gel and the location of the hybridizing sequence mapped by the Southern method (72). A comparison of singly digested DNA from clones 10, 35, 78 and 83 is shown in Figure 14. For each phage the sizes of restriction fragments observed as well as the corresponding hybridization pattern is distinct and nonoverlapping,

suggesting that each of these four phage carry a different bovine interferon gene. In addition, digestion of clone 83 with each of the three enzymes yields in each case two discrete hybridizing bands, indicating that this recombinant may carry two closely linked interferon genes.

F. Subcloning of the Bovine Leukocyte Interferon Genes

Restriction fragments from three of the recombinant phage which hybridized with the human leukocyte gene probe were subcloned into the multiple restriction enzyme cloning site of the pBR322 derivative, pUC9. The plasmid pUC9 was derived from pBR322 by first removing the 2,067 base-pair EcoRI-PvuII fragment containing the tetracycline resistance gene, then inserting a 425 base-pair HaeII fragment from the phage M13 derivative mP9 (78a) into the HaeII site of the resulting plasmid at position 2352 (relative to the pBR322 notation). The HaeII fragment from mp9 contains the N-terminal coding region of the E. coli lacZ gene in which a multi-restriction enzyme cloning site of the sequence, CCA AGC TTG GCT GCA GGT CGA CGG ATC CCC GGG, has been inserted between the 4th and 5th amino acid residues of β-galactosidase. Insertion of a foreign DNA fragment into these cloning sites disrupts the continuity between the lac promoter and lacZ gene, thus altering the phenotype of a JM83 transformed with the plasmid from lac^+ to lac^-.

The fragments referred to above were: (a) a 1.9 kb Bam HI fragment and 3.7 kb EcoRI fragment from clone 83 (which corresponds to nonoverlapping segments of the same recombinant), (b) a 3.5 kb BamHI-EcoRI fragment from clone 35, and (c) a 3.2 kb EcoRI fragment from clone 67. In each case, 0.1 micrograms of the appropriately digested vector was ligated with a tenfold molar excess of the purified fragment, transformed into E. coli strain JM83 (ATCC No. 39062, deposited March 5, 1982), plated out onto M9 (79) plates containing 0.04 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside and 0.2 mg/ml ampicillin. White colonies, which presumably carry a DNA insert at a restriction site interrupting the coding region of the lacZ gene on pUC9, were picked into 5 ml of LB broth plus

0.02 mg/ml ampicillin, grown for several hours at 37°C, and screened for the inserted fragment by a plasmid DNA minipreparation procedure (80).

G. DNA Sequence of a Bovine Leukocyte Interferon Gene on Clone 83

The DNA sequence extending from the Bam HI site of p83BamHI1.9kb (the 1.9 kb fragment subclone of clone 83) was determined by the Maxam-Gilbert chemical procedure (81), and is presented in Figure 15A. The longest open reading frame encodes a polypeptide of 189 amino acids with significant homology to the human leukocyte interferons. By analogy with the human proteins, the bovine leukocyte interferon consists of a hydrophobic 23 amino acid signal peptide which precedes a 166 amino acid mature protein by an identical sequence, ser-leu-gly-cys. Four cysteine residues at positions 1, 29, 99 and 139 are exactly conserved between species. As may be expected, the bovine protein is significantly less homologous (approximately 60 percent) to each of the human proteins than the latter are to one another (greater than 80 percent).

The DNA sequence and deduced amino acid sequence for an additional bovine leukocyte interferon gene occurring on the plasmid subclone p67EcoRI 3.2 kb are shown in Figure 15B.

H. Direct Expression of Mature BoIFN-α1 in E. coli

The construction of the direct expression plasmid is summarized in Figure 16. The plasmid subclone p83BamHI1.9kb was digested to completion with Ava II, and the 612 base-pair fragment containing the bovine leukocyte interferon gene isolated by electrophoresis on a 6 percent polyacrylamide gel and electroeluted. Approximately 1.5 micrograms of this fragment was digested with Fnu4H, phenol and ether extracted, and ethanol precipitated. The resulting Fnu4H sticky ends were extended to blunt ends with 6 units of DNA polymerase I (Klenow fragment) at 12°C for 30 minutes in 20 microliters containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 4 mM dithiothreitol and 0.1 mM each dATP, dGTP,

dCTP and dTTP. After extraction with phenol and ether, the DNA was digested with Pst I and electrophoresed on a 6 percent gel. The resulting 92 base-pair blunt end-Pst I fragment which extends from the first nucleotide of the coding region for the mature bovine leukocyte interferon was electroeluted from the gel.

The remainder of the mature coding region was isolated as follows. Three micrograms of the Bam HI insert of p83BamH11.9kb was partially digested with 14 units of Pst I for 10 minutes at 37°C in a 45 microliter reaction containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM dithiothreitol, and extracted with phenol and ether. The desired 1440 base-pair partial Pst I-Bam HI fragment extending from nucleotide 93 of the mature coding region was isolated from a 6 percent polyacrylamide gel.

The plasmid pdeltaRIsrc is a derivative of the plasmid pSRCex16 (82) in which the Eco RI sites proximal to the trp promoter and distal to the src gene have been removed by repair with DNA polymerase I (83), and the self-complementary oligodeoxynucleotide AATTATGAATTCAT (synthesized by the phosphotriester method (84)) was inserted into the remaining Eco RI site immediately adjacent to the Xba I site. 20 micrograms of pdeltaRIsrc was digested to completion with Eco RI, phenol and ether extracted, and ethanol precipitated. The plasmid was then digested with 100 units of nuclease S1 at 16°C for 30 minutes in 25 mM sodium acetate (pH 4.6), 1 mM $ZnCl_2$ and 0.3 M NaCl to create a blunt end with the sequence ATG. After phenol and ether extraction and ethanol precipitation, the DNA was digested with Bam HI, electrophoresed on a 6 percent polyacrylamide gel, and the large (4300 bp) vector fragment recovered by electroelution.

The expression plasmid was assembled by ligating together 0.2 micrograms of vector, 0.02 micrograms of the 92 bp blunt-Pst I fragment and 0.25 micrograms of the 1400 bp partial Pst I - Bam HI fragment with 400 units of T4 DNA ligase overnight at 12°C, and used to transform E. coli strain 294 (ATCC No. 31446) to ampicillin resistance. Plasmid DNA was prepared from 96 of the colonies and

digested with Xba I and Pst I. Nineteen of these plasmids contained the desired 103 base-pair XbaI-PstI and 1050 base-pair Pst I fragments. DNA sequence analysis verified that several of these plasmids had an ATG initiation codon correctly placed at the start of the bovine interferon coding region. One of these plasmids, pBoIFN-α1trp55, was chosen for further study.--

On page 22, line 3, change "pBI" to --pB1--; in line 9, after "plasmids." please insert:

--US 452,227 describes construction of the expression plasmid pR1:

a. Isolation of Messenger RNA for Prorennin

The fourth stomachs of freshly slaughtered calves of less than one week of age were removed and transported on ice from Conti Meat Packing Company, Inc., Henrietta, New York, and the mucosa promptly dissected away from the supporting tissue.

Polysomes were isolated from 217 grams of mucosa by the method of Palmiter, R.D., Biochemistry, 13: 3606 (1974), incorporated herein by reference, except: solutions were autoclaved for one half hour; tissue was homogenized with a phenol washed Brinkmann homogenizer (Polytron Model PT10ST), and polysomes were pelleted through 1M sucrose in PB (polysome buffer) in heat sterilized 250 ml Nalgene polycarbonate tubes.

The polysomes were then resuspended in 90 ml of 0.05M Tris, pH 7.2 plus 10 ml of Tenex extraction buffer (Tenex is 50 mM Tris, pH 7.0, 500 mM NaCl, 250 mM EDTA, 5 percent SDS), and the solution heated to 65°C.

Protein was removed by centrifugation at low speed to remove debris, followed by addition of 40 mg of Tritirachium aulbum proteinase K, bound to SAH-cellulose (MCB-EM reagent from MCB Manufacturing Chemists, Incorporated, Gibtown, N.J.). The mixture was incubated for one hour at 25°C with shaking, and the proteinase K-cellulose removed by centrifugation. The supernatant was extracted with an equal volume of 50:50 v/v phenol:chloroform. The mixture was centrifuged, and the aqueous phase extracted once with

chloroform, and the organic layer plus interphase bulk extract with 20 ml of 0.02M Tris, pH 7.2. The aqueous phases were combined and 0.1 volumes of 2M sodium acetate, pH 5.5, and two volumes of ethanol were added. The RNA was allowed to precipitate in storage at -80°C, and collected by centrifugation at 12,000 x g, lyophilized to remove residual ethanol, and redissolved in 10 mM Tris, 1 mM EDTA, pH 7.5.

This RNA solution was adjusted to an RNA concentration of about 2 mg per ml by diluting with 10 mM Tris, pH 7.4, 1 mM EDTA, 0.2 percent SDS, and 0.5 M NaCl, and passed over a column of oligo-dT-cellulose (Type 3, Collaborative Research, Lexington, Massachusetts) which had been prepared in 1.5 cm diameter sterile econo columns (Bio Rad Laboratories, Richmond, CA). The flow through was recycled over the column twice again. The column was then washed with binding buffer plus SDS until no UV absorbing material eluted as monitored by passing the eluate through a precision type 513 ultramicro continuous Quarasil flow cell in a Beckman L-25K spectrophotometer. The bound RNA was then removed from the column by washing with an elution buffer which had been warmed to 65°C and the eluate was collected in 2.5 ml fractions in sterile siliconized 13 x 100 borosilicate tubes using a Gilson microfractionator. The mRNA containing fractions were recycled over the oligo dT columns twice to achieve further purification of poly A messenger. The resulting poly A messenger fractions were brought to 0.2M in potassium acetate and precipitated with 2 volumes of ethanol and the precipitate stored at -80°C. This mRNA, unfractionated, was used to prepare cDNA as described below.

b. Materials and Methods for Construction of Expression Plasmids Incorporating Sequences from mRNA

Unless otherwise specified, the procedures used in the description herein are as follows:

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b.1 Preparation of cDNA from mRNA

The procedure is as described in Goeddel D. et al., Nucleic Acids Research, 8; 4057 (1980), incorporated herein by reference, and was applied with results subsequently to be described.

Briefly, for 100μg mRNA, the initial ^{32}P labeled single strand DNA was formed by placing the unfractionated mRNA, prepared as described above, in 100μl of reverse transcriptase buffer solution (20mM Tris, pH 8.3, 20mM KCl, 8mM $MgCl_2$, 30 mM BMSH) which is 1mM each in the four dNTPs, (100 microcuries ^{32}P dCTP), and contains approximately 2μg primer, 100 units RNAsin (Biotech) (an RNAase inhibitor) and 160 units of reverse transcriptase. The solution was incubated at 42°C for 30 minutes; then quenched in ice water and boiled for 3 minutes. After cooling, denatured protein was removed by centrifugation, and the supernatant recovered.

The complementary DNA chain was then formed by adding an equal volume of 1/2 times reverse transcriptase buffer with 10 units DNA polymerase Klenow fragment (Biolabs) and incubated at 14°C for 3 hours. Reaction was stopped and protein extracted by vortexing with 100μl of phenol saturated with buffer (5mM Tris, pH 8, 200mM NaCl, 1mM EDTA) and adding 100μl chloroform. The aqueous phase was then removed and the nucleic acid precipitated with 2.5 times the volume of ethanol at -20°C. The precipitate was spun down and washed with cold 70 percent ethanol. The foregoing method of protein removal by phenol chloroform and nucleic acid recovery by ethanol precipitation was followed throughout.

The pellet was then dissolved in 100μl SI buffer (25mM NaOAc, pH 4.5, 1mM $ZnCl_2$, 300mM NaCl) and treated with 800 units SI nuclease (Miles) at 37°C for 90 minutes to cleave the "hairpin" in the cDNA.

The mixture was then phenol-chloroform extracted as above set forth and nucleic acids precipitated using 4 volumes ethanol at dry ice temperatures.

The pellet was resuspended in 45μl TEB and 5μl 50 percent glycerol, 0.1 percent X C, 0.1 percent BPB added. The solution was

loaded onto 6 percent acrylamide gel prepared as described in Goeddel (supra) (5 ml 10x TEB, 2 ml 1.6 percent APS, 7.5 ml 40 percent acrylamide, 36 ml water, 50μl TEMED.) After 30 minutes at 300 volts the gel was cut and stained in EB to determine size locations. (Acrylamide gel electrophoresis was run in the foregoing manner throughout.) The band corresponding to the desired size was counted using Cerenkov counts and electroeluted into about 300μl 1/10 TEB buffer and recounted to determine yield of cDNA. The volume was reduced to 200μl, to this was added 20μl 3M NaOAc, pH 4.8 and 550μl cooled ethanol. This cDNA was stored at -20°C.

b.2 Colony Library Preparation

20 ng (as judged by recovered radioactivity) of the cDNA as prepared in paragraph b.1 was tailed with poly C as follows:

To 20ng cDNA, suspended in 10μl water, were added 10μl of 10x cacodylate buffer, 10μl 10mM dCTP, 68μl water, 1μl 100mM $CoCl_2$, and 10 units terminal transferase. The mixture was incubated at 37°C for 5 minutes and protein removed by phenol-chloroform extraction as described above. To supernatant was added 0.8 ng PstI cleaved pBR322 tailed with poly G, along with 10μl 3mM NaOAc, pH 4.8, and 275μl ethanol. The spun down (nucleic acid) precipitate was resuspended in 360μl water plus 40μl 10x annealing buffer (100mM Tris, pH 7.5, 2.5mM EDTA, 1M NaCl) and incubated at 70°C diminishing to 37°C overnight.

200μl of the annealed vectors thus prepared were used to transform 400μl of E. coli K12 strain 294 cells, ATCC 31466 by treating with calcium ion (calcium chloride solution). After 1 hour, the cells were heat shocked at 42°C for 70 seconds and held for 90 minutes in 5 ml LB. 400μl of cell culture was then plated onto 12 large plates of LB containing 5μg/μl Tc, and the transformed colonies were recovered.

The colonies were picked into microtiter dishes, incubated overnight, and stamped onto nitrocellulose filters (BA85) placed on LB plates containing 5μg per μl Tc. The remaining portions of the

colonies in the microtitered dishes were preserved by adding 25μl of 42 percent DMSO and storing at -20°C.

The transferred portions of the colonies were incubated for 8 to 9 hours at 37°C and amplified by transferring the filters to plates containing 12.5μg/ml chloramphenicol and incubating at 37°C overnight.

Each nitrocellulose filter containing transformed, cultured, and amplified colonies was probed for the desired cDNA sequence as follows:

Each filter was floated successively on a lysis solution (0.5M NaOH, 1.5M NaCl) for 3 minutes, on a neutralizing solution (0.5M Tris, 3M NaCl pH 7.5) for 15 minutes and on 2 x SSC solution for 15 minutes and then dried.

In the meantime, kinased probe (described below) was prepared by the method of Goeddel, et al., Proc. Nat. Acad. Sci., 76: 106 (1979), incorporated herein by reference. 100 pmol of DNA probe was treated in kinase buffer (60mM Tris, pH 8, 10mM $MgCl_2$, 10mM BME) with 25μl ^{32}P-ATP and 10 units of T4 kinase (BRL) for 30 minutes at 37°C. Labelled probe was separated from unincorporated ^{32}P using a G-50 column.

The dried filters described above were prehybridized by covering them with a solution of the following composition: 400 ml solution contained 72 ml 5M NaCl, 40 ml 50x Denhardt (50x Denhardt = 1 percent each bovine serum albumin, polyvinyl-pyrrolidine (PVP), and SDS), 20 ml 10 percent NP40, 80 mg sonicated salmon sperm DNA, 36 ml 1M Tris, pH 7.5, and 9.6 ml of 250 nM EDTA.

Labelled probe was then added and the filters were incubated at 37°C overnight. The filters were washed with 6x SSC with 0.1 percent SDS, dried, and placed against photographic film to locate colonies containing ^{32}P- i.e., those which are hybridized with probe.

b.3 Isolation of cDNA Plasmids and Sequence Analysis

Plasmids were isolated from the identified cultures using the cleared lysate method of Clewell, D.B. and Helinski, Biochemistry

9: 4428 (1970), incorporated herein by reference, and purified by column chromatography on Biorad A-50 Agarose. Smaller amounts (mini-preps) were prepared using the procedure of Birnboim, H.C. Nucleic Acids Research, 7: 1513 (1979).

Fragments of the cloned cDNA containing plasmids were prepared for sequencing by treating about 20 μg of plasmids with 10 to 50 units of the appropriate restriction enzyme or sequence of restriction enzymes in approximately 600 μl solution containing the appropriate buffer for the restriction enzyme used (or sequence of buffers); each enzyme incubation was at 37°C for one hour. After incubation with each enzyme, protein was removed and nucleic acids recovered by the phenol-chloroform extraction and ethanol precipitation as set forth above.

After cleavage, the preparation was treated for one hour at 37°C with 10 units Klenow in 100μl of Klenow buffer (50mM KPi, pH 7.5, 7mM $MgCl_2$, 1mM BME), containing 50 nmol dNTP. Protein was removed and nucleic acids recovered as above, and the nucleic acids suspended in 40μl of loading buffer for loading onto 6 percent polyacrylamide gel, as described above, for sizing.

DNA sequencing was performed by the M13 vector method (Messing, et al, Nucleic Acids Research, 9: 309 (1981)) or by the method Maxam, et al, Methods in Enzymology, 65: 499 (1980).

b.4. Ligation Procedures

DNA fragments, including cleaved expression plasmids, were ligated by mixing the desired components (e.g. vector fragment cut from 0.25 μg plasmid is mixed with insert cut from 1 μg of plasmid in 20 μl reaction mixture), which components were suitably end tailored to provide correct matching, with T4 DNA ligase. Approximately 10 units ligase were required for μl quantities of vector and insert components. The resulting plasmid vectors were then cloned by transforming E. coli K12 strain 294 (ATCC 31466). The transformation and cloning, and selection of transformed colonies were carried out as described above. Presence of the

desired sequence was confirmed by isolation of the plasmids from selected colonies, and DNA sequencing as described above.

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b.5 Additional Literature References:

Additional procedures for preparation of cDNA may be found in Goeddel, et al, Nature, 287: 411 (1980); Goeddel, et al, Nature, 281: 544 (1979); Wickens, et al, J. Biol Chem, 253: 2483 (1978).

c. Prorennin Expression

c.1 Preparation of Probes for Initial Cloning of the Prorennin in Sequence

Three types of probes were prepared as follows:

Four synthetic DNA sequences dTTCATCAT$\binom{A}{G}$TT$\binom{A}{G}$TC

were prepared by the method of Crea, R. and Horn, T., Nucleic Acids Res., 8: 2231 (1980) except that 2, 4, 6 - triisopropyl benzene sulfonyl - 3 - nitro - 1, 2, 4 - triazole (TPS-NT) was used as coupling agent (de Rooij, J.F.M. et al., Rec. Trav. Chin. Pays-Bas, 98: 537 (1979)). This set of probes will be referred to herein as the "probe mixture".

The sequence dGATCCGTCGAATTCGG, hereinafter called "primer/probe", was also prepared synthetically and utilized as indicated hereinbelow.

A third "Tth probe" was constructed by recovering and cloning the sequence between two Tth 111 I cleavage sites in a cloned plasmid derived from unfractionated mRNA. In preparing this probe, 100 μl of unfractionated mRNA, prepared as described above, was converted to cDNA, cloned, the plasmids recovered and the probe excised by treating with Tth 111 I. In this preparation, oligo-dT (12-18) was used as primer in the initial reverse transcriptase reaction; the cDNA was size separated on 6 percent acrylamide gel, and the fraction containing greater than 1000 base pairs was recovered for annealing into the cloning vector. Upon transforming E. coli 294, 800 transformed colonies were obtained, and one of them, 4H10, hybridized with "probe mixture". Plasmids were isolated from the 4H10 culture, and T7h111 I digestion carried out

as described above. The fragments were subjected to gel electrophoresis as described above, and the 320 base pair Tth 111 I "small" fragment electroeluted and labeled by boiling with 2μl (1 μg) of calf thymus primer in 28μl of water for 4 minutes, and adding the boiled solution to a tube containing 13μl of α-^{32}PdCTP (130μCi) (New England Nuclear), 9μl 0.1mM dGTP, dATP, dTTP, and 6μl of 10 x buffer (10 x buffer refers to 60mM NaCl, 60mM $MgCl_2$, 60mM Tris, pH 7.5, 50mM DTT). The reaction mixture was incubated at room temperature for 1 1/2 hours, and added to 40μl of 20 percent glycerol, 0.05 percent SDS, 20mM BBA, and loaded onto a G50 column to remove unincorporated nucleotides. The resulting labeled preparation was used as the "Tth probe".

c.2 Preparation of Prorennin Sequences

The prorennin gene for insertion into an expression plasmid was derived from a fragment at the 5' end ligated to a 3' end fragment. These were prepared as follows:

The 5' end fragment was a ligation of a synthetic fragment and a fragment derived from clone PFLA, as described below. The synthetic fragment has the structure:

[Met] [Ala] [Glu] [Ile] [Thr]

d A A T T C A T G G C A G A A A T A A C A A G

G T A C C G T C T T T A T T G T T C C T A G d

reading direction--->

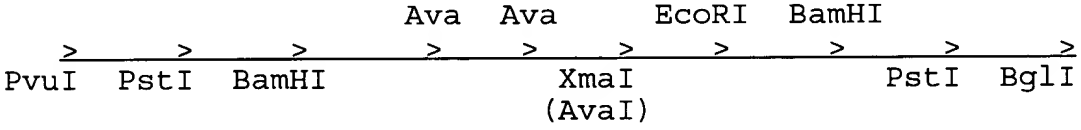
This sequence contains an EcoRI site at the "upstream end", an ATG start codon, followed by sequences coding for the first four amino acids in prorennin, and terminates in a BamH1 site.

The cloned fragment to which this synthetic fragment is ligated in order to form the 5' end of the gene comprises a Xma-BamHI fragment of approximately 440 base pairs derived from the 5' end of the gene.

The appropriate 440 bp fragment was obtained from cDNA derived from unfractionated mRNA as described above, using as primer, dGATCCGTCGAATTCGG, i.e., the "primer probe". The cDNA formed using

this primer was size fractionated as set forth above and fragments having more than 1,000 base pairs inserted into the PstI site of pBR322 for cloning. The resulting clones were selected using both Tth probe and the primer/probe as probes. Only colonies hybridizing with both were selected. From 1,280 transformed colonies, about 300 colonies were obtained which showed hybridization with both probes. These were examined for presence of the 5' portion of the prorennin sequence as follows:

The results of a series of double digestions using Ava I-Pvu I, AvaI-BamH I, Bgl I-BamH I, and Bgl I-EcoR I were analyzed. Advantage was thus taken of the known Pvu I and Bgl I sites, each 125 base pairs either side of the pBR322 Pst site utilized for insertion of the cDNA sequence. These digestions provide suitable fragments for analysis. A rough map of the appropriate fragment as it is disposed in the PstI site of pBR322 is shown below.



The desired clone, PFLA, was selected by analysis of acrylamide gel electrophoresis performed on the above double digests of mini preps prepared from the identified clones. Plasmids were then isolated from PFLA clone, double digested with BamH I and XmaI, and the 440 bp fragment recovered by gel electrophoresis.

The "complete" 5' end was then created by a standard ligation reaction utilizing the synthetic fragment and the PFLA clone BamHI-XmaI fragment with T4 ligase followed by cleavage with XmaI and EcoRI. The resulting ligated sequences were purified on acrylamide gel electrophoresis selecting for the appropriate 455 base pair fragment.

The 3' end fragment was prepared in a manner analogous to that used to prepare the PFLA clone. cDNA containing >1000 bp formed from unfractionated messenger RNA using oligo-dT as primer, was cloned as above, and colonies selected with Tth probe.

Approximately 50 colonies resulted. The desired clone was selected by analyzing the results of gel electrophoresis formed on plasmid minipreps which were double digested with BamHI/BglI, PvuI/BamHI, EcoR1/BglI, and PvuI/EcoR I again taking advantage of the PvuI and BglI sites flanking the PstI insert site. The plasmids from the desired colony were isolated then cleaved with Xma I and Pst I and electrophoresed to isolate the 800 bp sequence of the "3-375" fragment shown in Figure 17. The "3-375" fragment extends from the Xma site congruent with that from the PFLA fragment, past the end of the gene to the Pst I insertion site. An approximate map of this fragment as it is deployed in the PstI site of pBR322 is shown below.

PvuI PstI EcoRI BamHI BamHI PstI BglI
> > > > > > > >
XmaI

c.3 Construction of the Expression Plasmid pR1

Figure 17 outlines the construction of the initial plasmid used for prorennin expression. Advantage is taken of a hybrid gene at the 5' end in which the section coding for the N-terminal portion of the prorennin is made synthetically, thus allowing the incorporation of an ATG initiation codon for protein synthesis in front of the triplet coding for the first amino acid.--

On page 22, line 10, please change "Imm" to --IMM-- and after "with" insert --the--; in line 11, after "segments" insert --constructed as described above--; in line 12, after "plasmid" insert --such that expression is under control of the E. coli trp promoter as--; in lines 12-13, change "filed Oct. 19, 1981" to --supra,--; in line 13, change "Supra." to --supra.-- Transcription is in the direction of the tetracycline resistance gene of this plasmid. The ligation mixture was used to transform E. coli K12 294.

C1

Plasmids were isolated from the selected transformed clones, and one was selected for sequencing to confirm the proper junction of the desired fragments.--

On page 32, after line 23, please insert:

--63. Blin and Stafford Nucleic Acids Research 3, 2303 (1976).

C8

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69. Goeddel et al., Nature 290, 20 (1981).

70. Goeddel et al., Nucleic Acids Research 8, 4057 (1980).

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78a. Messing et al., Nucleic Acids Research 9, 309 (1981).

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